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The Mechanism of Hydrolysis of β-Glycerophosphate by Kidney Alkaline Phosphatase

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1. To identify the functional groups that are involved in the conversion of β-glycerophosphate by alkaline phosphatase (EC 3.1.3.1) from pig kidney, the kinetics of alkaline phosphatase were investigated in the pH range 6.6–10.3 at substrate concentrations of 3 μM–30 mM. From the plots of $\log \bar{V}^{H^+}$ against pH and $\log \bar{V}^{H^+}/\bar{K}_m^{H^+}$ against pH one functional group with $pK = 7.0$ and two functional groups with $pK = 9.1$ were identified. These groups are involved in substrate binding. Another group with $pK = 8.8$ was found, which in its unprotonated form catalyses substrate conversion. 2. GSH inhibits the alkaline phosphatase reversibly and non-competitively by attacking the bound Zn(II). 3. The influence of the H^+ concentration on the activation by Mg^{2+} ions of alkaline pig kidney phosphatase was investigated between pH 8.4 and 10.0. The binding of substrate and activating Mg^{2+} ions occurs independently at all pH values between 8.4 and 10.0. The activation mechanism is not affected by the H^+ concentration. The Mg^{2+} ions are bound by a functional group with a pK of 10.15. 4. A scheme is proposed for the reaction between enzyme, substrate, Mg^{2+} and H^+, and the overall rate equation is derived. 5. The mechanism of substrate binding and splitting by the functional groups of the active centre is discussed on the basis of a model. Mg^{2+} seems to play a role as an autosteric effector.

Investigations on the number and structure of the active sites of alkaline phosphatase (EC 3.1.3.1) have been carried out largely with the enzyme from *Escherichia coli* (Trentham & Gutfreund, 1968; Halford *et al.*, 1969; Fernley & Walker, 1969; Petitclerc *et al.*, 1970). Principally two methods have been used to identify the functional groups in the active site: studies of the influence of pH on the kinetic constants K_m and V (e.g. Lazdunski *et al.*, 1969), and the application of group-specific inhibitors (e.g. Fishman & Ghosh, 1967).

Previously published studies on the functional groups of alkaline phosphatase are to some extent contradictory. Some authors (Krishnaswamy & Kenkare, 1970; Gottesmann *et al.*, 1969) concluded that an imidazole group of histidine is present in the active site. Others (Siebert *et al.*, 1965; Fishman & Ghosh, 1967; Brunel *et al.*, 1969) have postulated the participation of the ε-amino group of lysine. It has also been suggested that a tyrosine residue occurs in the active site (Sizer, 1942; Hiwada & Wachsmuth, 1974). The results of investigations on the occurrence of –SH groups in the active site of alkaline phosphatase are also not in complete agreement. It has been reported that the alkaline phosphatase from *E. coli* contains no —SH groups (Schlesinger & Barrett, 1965; Simpson *et al.*, 1968). On the other hand, the alkaline phosphatase from intestine was reported to be inhibited by thiol-specific reagents (Lazdunski & Quellet, 1962; Fishman & Ghosh, 1967). Fosset *et al.* (1974) found only a reaction of calf intestine alkaline phosphatase after the enzyme had been denatured.

It is one aim of the present work to furnish a more exact answer to the question of the pK values of the functional groups in the active site of alkaline phosphatase. The mechanisms between enzyme, substrate and Mg^{2+} discussed so far are generally derived for a distinct pH value, in most cases for the pH optimum (e.g. Ahlers, 1974). In some cases, the reaction mechanism is pH-dependent and hence the substrate conversion proceeds via different ways depending on the pH value. For this reason, the effects of pH value on the substrate conversion are investigated in this paper. In addition, we were interested in the influence of the Mg^{2+} concentration on the pK values of the functional groups. Further, we wanted to evaluate the pK values of the amino acid residues that are involved in the activator-binding process. This can be done by varying the Mg^{2+} concentration; this is analogous to varying the substrate concentration, which can be used to estimate the functional groups that are involved in substrate binding and splitting.

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Experimental

Materials

Reagent-grade chemicals were obtained from E. Merck A.G., Darmstadt, Germany. For column chromatography DEAE-cellulose (Serva-Entwicklungslabor, Heidelberg, Germany) and Sephadex (Pharmacia, Uppsala, Sweden) were used.

Methods

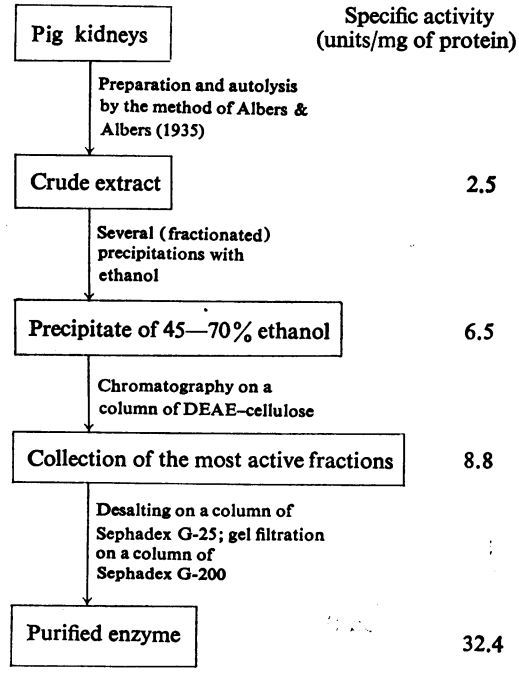
Purification of alkaline phosphatase. Alkaline phosphatase was obtained from pig kidney. The method of preparation and purification is shown in Scheme 1. By adding 0.5mM-Mg^{2+} ions and by maintaining a constant Zn^{2+} concentration with a nitrilotriacetate–Zn^{2+} buffer (150μM-nitrilotriacetate +50μM-$ZnCl_2$) at all steps of the preparation it was possible to stabilize the enzyme completely. Thus kinetic measurements could be made under reproducible conditions. To perform a large number of kinetic measurements under constant conditions it is more important to have large stabilized enzyme preparations than small preparations with a higher activity. Thus we did not attempt further purification and the specific activity of the alkaline phosphatase is therefore considerably lower than that obtained by Wachsmuth & Hiwada (1974), who carried out several more purification steps.

Fractionation. Ethanol (70%, v/v) was added to the crude extract at 2°C. The precipitate was washed twice with cold 70% ethanol and then dissolved in 50mM-Na_2CO_3–$NaHCO_3$ buffer, pH9.6, containing Mg^{2+}, Zn^{2+} and nitrilotriacetate at the concentrations stated above. Ethanol was then added at 2°C in steps of 5% (v/v). Each precipitate was dissolved as described above and the most active fractions were collected.

Chromatography procedures. DEAE-cellulose chromatography was performed on a column (15mm× 200mm), which was equilibrated with 10mM-$ZnCl_2$ and 10mM-Tris buffer, titrated to pH7.9 with 1M-acetic acid. Elution was performed with the same buffer, containing 0, 5 and 25mM-magnesium acetate. Desalting was performed on a column (20mm× 360mm) of Sephadex G-25, equilibrated with 10mM-$ZnCl_2$. Gel filtration was performed on a column (20mm×800mm) of Sephadex G-200, equilibrated with 10mM-$ZnCl_2$. The eluent was 10mM-Tris (titrated to pH7.9 with acetic acid)–100mM-NaCl–10nM-$ZnCl_2$.

Determination of enzyme activity. Enzyme activity was determined as described by Ahlers (1974), with the use of 10mM-β-glycerophosphate in the standard assay. By continuously recording the amount of phosphate released, it could be established with certainty that the progress curves were linear over the period required for the determination of initial rate. At β-glycerophosphate concentrations of 10μM or below, the amount of substrate hydrolysed exceeded 10%. Thus the activity was determined by drawing the tangent at the time–product curve at $t=0$. Because of the continuous recording these results were reproducible with an error of less than 10%. Under optimum conditions the error was less than 3%.

For the series of plots of [S] versus pH, the test medium contained different amounts of enzyme and substrate, 10μM-nitrilotriacetate, 5μM-$ZnCl_2$, 1mM-$MgCl_2$ (at this concentration the enzyme is approximately saturated by Mg^{2+} ions) and the appropriate buffer. For pH values below 8.8, 32mM-Tris was adjusted to the desired pH by the addition of 1M-HCl. For pH values above 8.8, 12mM-glycine was adjusted to the desired pH with 0.2M-NaOH. The pH value was determined immediately after the start of the reaction. The concentration of the buffer was kept as low as possible to minimize the error that could possibly be incurred by the transfer of the P_i residue to Tris. The use of the metal buffer system nitrilotriacetate–$ZnCl_2$–$MgCl_2$ served a double purpose. First, heavy-metal contaminants which could have exerted inhibitory effects, were



Scheme 1. *Purification of alkaline phosphatase*

Further details are given in the text. One unit of activity is defined as 1μmol of substrate transformed/min at 30°C.

chelated; secondly the Zn^{2+} concentration could be adjusted to 2.5pM and varied only slightly with pH changes (Wolf, 1973). Further, the excess of Mg^{2+} ions used ensured that measurements were always made at the Mg^{2+} concentration optimum. In the experiment designed to examine the influence of Mg^{2+} ions on the pK values the test medium differed slightly. It contained enzyme (at various concentrations so that measurements could be made under optimum conditions), substrate (20μM–20mM-β-glycerophosphate), $MgCl_2$ (4.35μM–3.15mM), buffer (for pH values below 9, 32mM-sodium borate was used, adjusted to the desired pH value with 1M-HCl; for pH values above 9, 100mM-Na_2CO_3 was used, adjusted to the desired pH with 1M-HCl), nitrilotriacetate (4mM) and $ZnCl_2$ (2mM). The nitrilotriacetate–Zn^{2+}–Mg^{2+} buffer system was used to adjust both the Mg^{2+} and Zn^{2+} concentrations to the desired values ([Mg^{2+}] = 0.1μM–1.15mM; [Zn^{2+}] = 2.5pM). The calculated values for the Mg^{2+} concentration were corrected for the pH-dependence of the dissociation constant of the nitrilotriacetate–Mg^{2+} complex, and for the Mg^{2+} impurities of the reagents used, but not for the Mg^{2+} concentrations of the enzyme preparation, which were unknown. This error could possibly be considerable at low pH values, because of the increased amount of enzyme used in the experiments and because of the increased dissociation constant for nitrilotriacetate–Mg^{2+}.

Determination of protein concentration. Protein was generally determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. In some cases the absorption at 280nm was used to measure the protein concentration.

Derivation of the reaction velocity equation. The rate equations were derived from the rapid-equilibrium reaction mechanism according to models presented by Botts & Morales (1953), Laidler (1956), Ohlenbusch (1962) and Cleland (1970). Methods used to interpret the results of varying pH have also been described ((Dixon, 1953; Alberty, 1956; Laidler, 1958; Dixon & Webb, 1965; Ahlers *et al.*, 1974).

Observations on enzyme stability. The theory of pH influence which is applicable in the present work is exclusively concerned with reversible changes. To ensure that these requirements were met and to eliminate possible irreversible influences, measurements of enzyme stability were made at various pH values (results not shown). From these control experiments it was apparent that the alkaline phosphatase is stable between pH7 and 10 at room temperature as well as at 30°C. Above pH10 at 30°C a small loss in activity is observed and below pH7 at 30°C a larger loss of activity is observed.

Influence of GSH. The assay medium contained 50mM-Na_2CO_3–$NaHCO_3$ buffer, pH9.6, 10mM-$MgCl_2$ and enzyme. GSH (2mM) was added to a portion of the test medium. GSH (2mM) was added to a second portion of the test medium and then after 30min incubations, 0.5mM-$ZnCl_2$ was added. After 60min the activity of all the samples was determined by the addition of 10mM-β-glycerophosphate. The same substances were used in the assay for the determination of the type of inhibition except that GSH was present at a concentration of 0.4–4mM. After 15min incubation the reaction was started by the addition of 0.5–20mM substrate.

Symbols and abbreviations

E, EH, EH_2, free enzyme with various degrees of protonation; $[E]_t$, total enzyme concentration; S, substrate (sodium β-glycerophosphate); ES, EH_2S, EH_3S, enzyme–substrate complex with various degrees of protonation; k, reaction rate constant; k_{+1}, k_{-1}, k_{+2}, rate constants of the following enzyme–substrate reactions:

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E+\text{products}$$

K, dissociation constant; $K'_m = (k'_{-1}+\beta k_2)/k'_{+1}$, modified Michaelis constant; v, reaction rate under various experimental conditions at any substrate concentration; V, reaction rate where the enzyme is saturated with substrate and activator; $\bar{K}_m^{H^+}$, $\bar{V}^{H^+}$, apparent constants, which depend on H^+ ion concentration; $\bar{V}^{H^+,Mg^{2+}}$, maximum reaction rate ([S] tends to infinity) dependent on the H^+ and Mg^{2+} concentrations; K''_a, dissociation constant of the functional group, which dissociates in the EH_2 and EH_2S species and which is involved in the Mg^{2+} binding. The other constants and symbols are explained in the text.

Estimation of error

Kinetic constants were determined graphically. In the reciprocal plots the error was determined by drawing the boundaries of the linear plots, and the error of the kinetic constants was taken as the maximum obtained. The errors that occur when $\log \bar{V}^{H^+}$ or $\log(\bar{V}^{H^+}/\bar{K}_m^{H^+})$ is plotted against pH lead to errors in the pK values, which are given in the Results and Discussion section.

Results and Discussion

pK values of the functional groups

By varying pH and [S] it is possible to determine the pK values of the functional groups that are involved in the binding and conversion of the substrate and it is also possible to distinguish between them (Dixon, 1953; Alberty, 1956; Laidler, 1958; Dixon & Webb, 1965; Ahlers *et al.*, 1974).

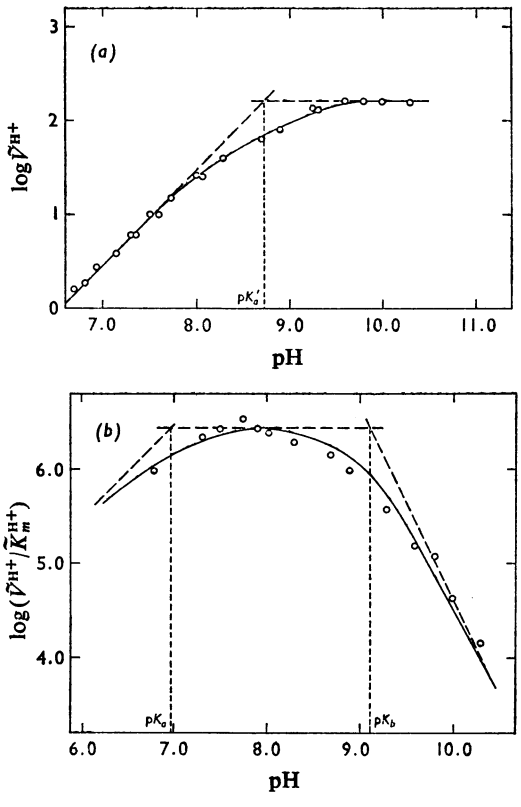


Fig. 1. *Plot of* log $\tilde{V}^{H+}$ *versus pH* (*a*) *and log* ($\tilde{V}^{H+}/\tilde{K}_m^{H+}$) *versus pH* (*b*)

[Mg^{2+}] = 1 mM; temperature = 30°C; for pH values below 8.8, 32 mM-Tris was adjusted to the desired pH value with 1 M-HCl; above pH 8.8, 12 mM-glycine was adjusted to the desired pH value with 1 M-NaOH. $\tilde{V}^{H+}$ and $\tilde{K}_m^{H+}$ were determined from the ordinate and abscissa intercepts of plots of $1/v$ versus 1/[S]. ○, Measured values; ——, theoretical curves drawn according to the theory described by Dixon & Webb (1965), taking into account that they miss the intersection points of their asymptotes (----) by a vertical distance of log 2 or of log 3, if two groups are involved with similar pK values.

It is necessary to determine the pH-dependence of V and K_m over the largest possible range of measurements. Substrate concentrations between 3 μM and 30 mM were used in conjunction with pH values between 6.6 and 10.3. The lower range was limited by decreased stability and much lower alkaline phosphatase activity. The upper range was limited by the precipitation of $Mg(OH)_2$. The v values were related to the same enzyme concentration. For measurements above pH 10 and below pH 7 a correction was made based on the measurements of enzyme stability. Non-enzymic hydrolysis of the substrate was less than 1% at all pH values and was therefore ignored. The data for the reaction rate v are given in μmol/min.

The results were plotted as $1/v$ versus 1/[S] for the various pH values (results not shown), and from the resulting linear plots $\tilde{K}_m^{H+}$ and $\tilde{V}^{H+}$ were determined by the method of Lineweaver & Burk (1934). In addition, at high pH values the plot of $v = f(v/[S])$ was used. In Figs. 1(*a*) and 1(*b*) log $\tilde{V}^{H+}$ versus pH and log($\tilde{V}^{H+}/\tilde{K}_m^{H+}$) versus pH are plotted. From the plot of log $\tilde{V}^{H+} = f$(pH) the pK values of the functional groups involved in substrate splitting could be determined.

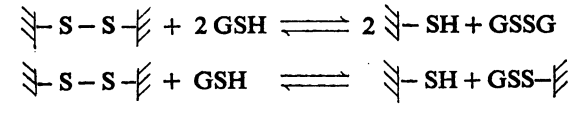
From Fig. 1(*a*) it is apparent that the maximum velocity increases over the pH range 6.6–8.8. The slope equals +1. Above pH 8.8 $\tilde{V}^{H+}$ does not change. (Here linear extrapolations of the origin and plateau of the curves were used and not the actual values plotted.) From this it follows, provided that the rate-determining step does not change with pH, that the reactive form of the enzyme–substrate complex contains a group of pK 8.8±0.1 at 30°C in its non-protonated form.

In plots of the log($\tilde{V}^{H+}/\tilde{K}_m^{H+}$) versus pH bends in curves produced by functional groups active in the ES complex are obliterated. Thus only bends produced by pK values of functional groups involved in substrate binding occur.

Dissociation of a functional group with a pK value of 7.0 makes substrate binding possible (Fig. 1*b*). There are also two groups involved in substrate binding with pK of 9.1±0.1. They are active in the protonated form. Above pH 9.1 they are largely deprotonated and can no longer bind the substrate. A change in the dissociation of β-glycerophosphate cannot occur at pH 7.0 or 9.1 since the dissociation constants of this compound are $K_1 = 43$ mM and $K_2 = 0.46$ μM (Kiesling, 1934).

Influence of GSH

GSH can participate in two different reactions: (1) the reaction with disulphide bridges:



(2) the reaction with the essential zinc atoms in the enzyme. In the case of a reaction of GSH with the essential zinc atoms, a reactivation by the addition of Zn^{2+} ions should be possible. Table 1 shows that the addition of GSH decreases the activity. The velocity of inactivation is very fast (less than 2 min) and thus the time-course of inactivation could not be followed by the method used (Ahlers, 1972). By adding $ZnCl_2$ considerable reactivation

Table 1. *Inhibition by GSH and reactivation with Zn^{2+} ions*

The samples were incubated for 60min at 30°C in 50mM-Na_2CO_3–$NaHCO_3$ buffer, pH9.6, and 10mM-$MgCl_2$. In one case $ZnCl_2$ was added after 30min. After 60min total incubation time enzyme activity was determined as given in the Experimental section.

Sample	[GSH] (mM)	[Zn^{2+}] (mM)	Relative activity (%)
1	—	—	100
2	2	—	31
3	2	0.5	85

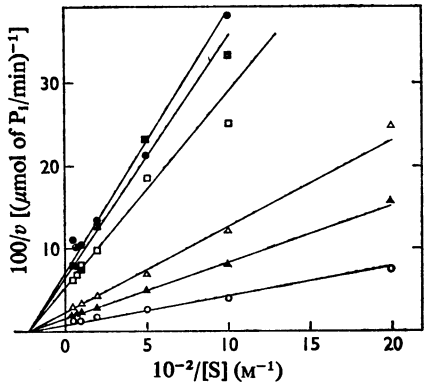
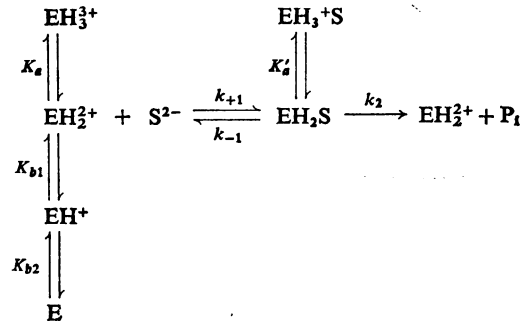


Fig. 2. *Plot of $1/v$ versus $1/[S]$ for various concentrations of GSH*

$[MgCl_2] = 10mM$; pH = 9.6 (50mM-Na_2CO_3–$NaHCO_3$ buffer); temperature = 30°C. [GSH]: ■, 4mM; ●, 2mM; □, 1.33mM; △, 0.6mM; ▲, 0.4mM; ○, no GSH added.

was achieved. It is probable that complete reactivation was precluded since the concentration of free Zn^{2+} used in this case effects a partial inhibition of the alkaline phosphatase (Ackermann, 1972).

The speed of inactivation and the reactivation by addition of Zn^{2+} ions make reaction (1) above less probable. Further support for the assumption that GSH inactivates the alkaline phosphatase by a reaction with Zn(II) is given by the results obtained by varying the GSH concentration in the absence and presence of a small amount of nitrilotriacetate–Zn^{2+} buffer (20μM-nitrilotriacetate–10μM-$ZnCl_2$). In the absence of nitrilotriacetate–Zn^{2+} half-maximum activity is obtained at 0.5mM-GSH and in the presence of nitrilotriacetate–Zn^{2+} it is obtained with 3mM-GSH. For reaction (1) this small amount of nitrilotriacetate–Zn^{2+} buffer should not influence the reaction significantly; for reaction (2) these results can be explained by the different equilibrium constants: $pK_{\text{nitrilotriacetate-Zn}}$ 10.6; $pK_{\text{GSH-Zn}}$ 8.3 (Sillén & Martell, 1964). This result agrees with a



Scheme 2. *Reaction of enzyme, substrate and H^+ ions*

$pK_a = 7.0$, $pK_b = 9.1$, $pK'_a = 8.8$.

series of papers (Lazdunski *et al.*, 1969; Gottesmann *et al.*, 1969; Krishnaswamy & Kenkare, 1970; Hirnberg & Laidler, 1973) in which evidence for the participation of Zn^{2+} ions in the catalytic process has been described. To determine the type of inhibition involved, substrate concentrations were varied at various GSH concentrations. Fig. 2 shows that straight lines with a common point of intersection on the abscissa were obtained when $1/v$ was plotted against $1/[S]$. As the inhibition by GSH is spontaneous and probably completely reversible the inhibition is non-competitive.

Derivation of a reaction rate equation

By using the pK values of functional groups of alkaline phosphatase obtained from Figs. 1(*a*) and 1(*b*) one can derive the reaction between enzyme, substrate and H^+ ions (Scheme 2). By assuming equilibrium conditions, one obtains from Scheme (2) the following reaction rate equation:

$$v = \frac{V \cdot [S]/(1+[H^+]/K'_a)}{K_m \cdot \dfrac{(1+[H^+]/K_a+K_b/[H^+]+K_b^2/[H^+]^2)}{(1+[H^+]/K'_a)} + [S]} \quad (1)$$

When plots of the logarithm of the kinetic constants versus pH are made for alkaline phosphatase from *E. coli* (Lazdunski *et al.*, 1969; Gottesmann *et al.*, 1969; Krishnaswamy & Kenkare, 1970), calf intestine (Morton, 1957; Fernley & Walker, 1965; Fosset *et al.*, 1974), chick intestine (Hirnberg & Laidler, 1973) rat liver (Melani *et al.*, 1967) and pig kidney (Hiwada & Wachsmuth, 1974) the curves obtained are similar to those obtained in the present paper (Figs. 1*a* and 1*b*). In these cases, an increase of $\log \hat{V}^{H+}$ up to a point of saturation and/or a decrease in the $p\hat{K}_m^{H+}$ value with increasing pH was observed. For the pK values of the identified functional groups,

there are, however, apparently very few similarities between the different enzymes. This could be due to a number of reasons. Frequently measurements were made over too small a pH range (Morton, 1957; Fernley & Walker, 1965; Melani *et al.*, 1967). The source of the enzyme also appears to have a strong influence on the results; the functional groups occurring in the active site could vary from species to species. It is, however, more probable that the amino acid sequence in the vicinity of the active site can vary and that the resulting variable charge distributions lead to a shift of the dissociation constants of the functional groups. A third possible reason for the different pK values could be due to measurements being made under different conditions of buffer composition, ionic strength and Mg^{2+} concentration. This is particularly problematical if several different buffers must be used within a given series of measurements (Ahlers, 1972).

In spite of these difficulties, there are a number of similarities which become apparent when the pK values of functional groups of alkaline phosphatases from various mammalian tissues are determined. Morton (1957), Siebert *et al.* (1965) and Hirnberg & Laidler (1973) found a functional group with pK8.6–9.2 when studying alkaline phosphatase from calf and chicken intestine. This group is active in the protonated form and involved in substrate binding in a similar way to those groups with p$K = 9.1$ which occur in alkaline phosphatase from pig kidney. There are, however, larger differences apparent in the case of the pK value of the functional group which dissociates in the ES complex. Hiwada & Wachsmuth (1974) reported pK values of 7.9 and 8.7 for the free enzyme and 8.7 for the ES complex of alkaline phosphatase from pig kidney. These results partially agree with our findings. We also found a group with pK8.8 dissociating in the ES complex. However, our results suggest the presence of one group with pK7.0 and two groups with pK9.1 dissociating in the free enzyme. These differences may be a consequence of the fact that Hiwada & Wachsmuth (1974) did not interpret their results exactly according to the theory given by Dixon (1953) and Dixon & Webb (1965). According to this theory the curvature at the bends in the logarithmic plots is such that the graph misses the intersection point of the neighbouring straight parts by a vertical distance of log2: if two pK values occur together the distance is equal to log3. If Hiwada & Wachsmuth (1974) had constructed the asymptotes in that way the calculated pK values would have been more like those reported in the present paper.

Further, there is a smaller number of measurements at high pH in the paper of Hiwada & Wachsmuth (1974). Thus, at least from their measurements with β-glycerophosphate, a straight line with a slope of -2 in the plot of $\log(V/K_m)$ versus pH can be drawn as well, resulting in two groups with a pK of about 9, which agrees with the interpretation in the present paper. Also, for the descending part of the curves at low pH values only two experiments were carried out, so that the resulting pK may be lower than 7.9. Also the pK of 7.0 from Fig. 1(*b*) in the present paper may not be very exact, as there are also only two points on the descending part of the curve.

pH-dependence of the activation by Mg^{2+} ions

To investigate whether the pH affects the mechanism of the reaction between enzyme, substrate and Mg^{2+} ions, and whether varying the Mg^{2+} concentration has any influence on the pK value, measurements at 30°C were made at various concentrations of substrate, H^+ and Mg^{2+} ions. Since three of the four functional groups have pK values of about 9, the measurements were made in a pH range of 8.36–10.03. The influence of Mg^{2+} ions on the functional group with a pK of 7.0 was not investigated because of the rather low activity of the alkaline phosphatase within this pH region, which would have led to considerable error. The results are presented for each pH value in a plot of $1/v$ versus $1/[Mg^{2+}]$ for a series of different substrate concentrations. In Fig. 3 the data are shown for pH8.99 and in Fig. 4 for pH10.03. From these plots the apparent values of $\tilde{V}^{H^+,S}([Mg^{2+}]\rightarrow\infty)$ were obtained from the ordinate intercepts of the straight lines. For each pH value $1/v$ was plotted against 1/[S] at various Mg^{2+} concentrations. An example is given for pH8.99 in Fig. 5 and for pH10.03 in Fig. 6. These Figures also contain the values for

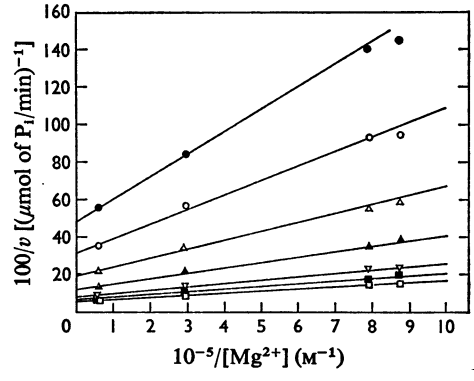


Fig. 3. *Example of the $1/v$ versus $1/[Mg^{2+}]$ plots of the alkaline phosphatase reaction rate at variable concentrations of Mg^{2+}, H^+ and β-glycerophosphate*

The Mg^{2+} concentration was adjusted with 4mM-nitrilotriacetate; pH = 8.99 (32mM-sodium borate buffer). [β-Glycerophosphate]: ●, 40μM; ○, 60μM; △, 100μM; ▲, 200μM; ▽, 400μM; ■, 600μM; □, 1mM.

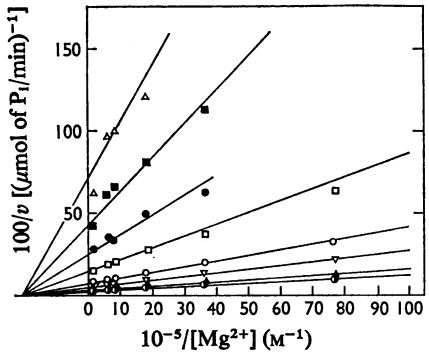


Fig. 4. *Example of the* 1/*v versus* 1/[Mg^{2+}] *plots*

pH = 10.03 (100mM-Na_2CO_3–$NaHCO_3$ buffer). The Mg^{2+} concentration was adjusted with 4mM-nitrilotriacetate. [β-Glycerophosphate]: △, 400μM; ■, 600μM; ●, 1mM; □, 2mM; ○, 4mM; ▽, 6mM; ▲, 10mM; ◑, 20mM.

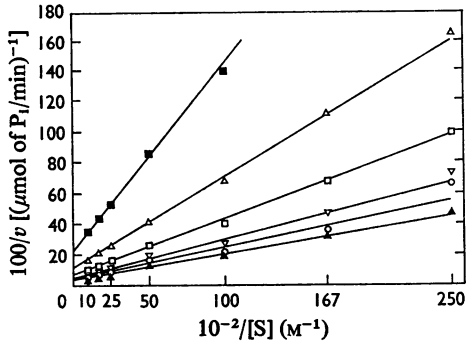


Fig. 5. *Example of the* 1/*v versus* 1/[*S*] *plots of the reaction rate at variable concentrations of β-glycerophosphate,* H^+ *and* Mg^{2+}

pH = 8.99 (32mM-sodium borate buffer). [Mg^{2+}]: ■, 0.4μM; △, 1.0μM; □, 2.5μM; ▽, 6.3μM; ○, 16μM; ▲, infinity. The Mg^{2+} concentration was adjusted with 4mM-nitrilotriacetate.

[Mg^{2+}] → ∞ obtained from Figs. 3 and 4. Both types of plots shown in Figs. 3–6 yield an intercept of all straight lines in the second quadrant near the abscissa. Analogous plots were made for each pH value at which measurements were taken (results not shown).

As discussed by Ahlers (1974) these plots can be described by eqn. (2):

$$\frac{1}{v} = \frac{1}{V} + \frac{K'_m \cdot K_{Mg}}{V}\left(\frac{1}{[S]} + \frac{1}{K_s}\right)\frac{1}{[Mg^{2+}]} + \frac{K'_m}{V}\cdot\frac{1}{[S]} \qquad (2)$$

From the common points of intersection and from secondary plots the kinetic constants can be calculated (Ahlers, 1974). They depend on the pH value. In analogy with the plot of $p\tilde{K}_m^{H+}$ versus pH, which provides data on the functional groups responsible for substrate binding and conversion, the plot of $p\tilde{K}_m^{H+}$ versus pH shows the pK values of functional groups involved in binding of Mg^{2+} to the enzyme and in substrate conversion.

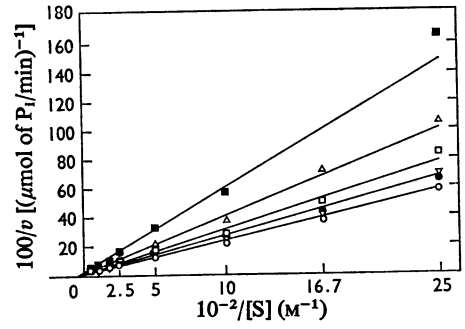


Fig. 6. *Example of* 1/*v versus* 1/[*S*] *plots*

pH = 10.03 (100mM-Na_2CO_3–$NaHCO_3$ buffer). [Mg^{2+}]: ■, 0.4μM; △, 1.0μM; □, 2.5μM; ▽, 6.3μM; ●, 16μM; ○, infinity. The Mg^{2+} concentration was adjusted with 4mM-nitrilotriacetate.

In the equation $K_{Mg} = k_{-Mg}/k_{+Mg}$, k_{-Mg} is the rate constant for the decomposition of the EH_2Mg complex (Scheme 3), whereas k_{+Mg} is the rate constant for the combination of EH_2 and Mg^{2+}. The pH-dependence of both rate constants is given by the Michaelis–pH functions of the EH_2Mg and the EH_2 complexes respectively:

$$p\tilde{K}_{Mg}^{H+} = p\left(\frac{k_{-Mg}}{f_{EH_2Mg}}\cdot\frac{f_{EH_2}}{k_{+Mg}}\right) = pK_{Mg} + pf_{EH_2} - pf_{EH_2Mg} \qquad (3)$$

Since substrate and activator are bound by the enzyme independently (Ahlers, 1974) the pH-dependence of $\tilde{K}_{Mg}^{H+}$ is equal to that of $\tilde{K}'^{H+}_{Mg}$:

$$p\tilde{K}'^{H+}_{Mg} = pK'_{Mg} + pf_{EH_2S} - pf_{EH_2MgS} \qquad (4)$$

Eqn. (4) shows that the dissociation of a group of the EH_2MgS complex yields an upward-directed bend and the dissociation of a group of the EH_2S complex, responsible for binding of the activator, yields a downward-directed bend in the $p\tilde{K}_{Mg}^{H+}$ versus pH plot. Fig. 7 shows the pH-dependence of $p\tilde{K}_{Mg}^{H+}$, obtained from plots of 1/v versus 1/[Mg^{2+}]. From this Figure the presence of two functional groups can be derived. One of them dissociates in the EH_2MgS complex with a pK value of 9.1±0.1. It is probably identical with a pK8.8 group, which catalyses the substrate conversion and which was described above. At pH values above 10 there is a second group, which is involved in the activator-binding process. Since Mg^{2+} ions are essential for the conversion of the substrate, V is determined by the dissociation state of two functional groups. Hence the bend of the log $\tilde{V}^{H+}$ versus

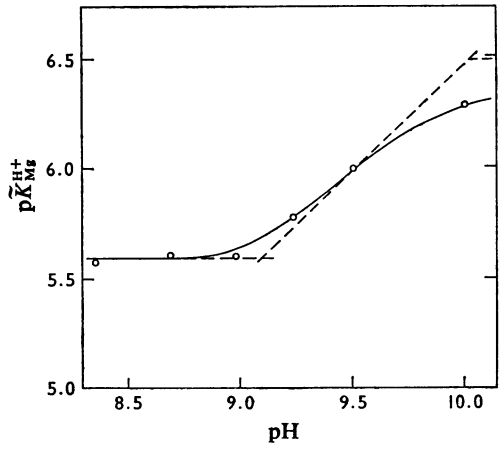


Fig. 7. *$p\tilde{K}^{H+}_{Mg}$ versus pH plots for evaluation of the ionizable groups of the enzyme–substrate–Mg^{2+} complex and of the groups that bind Mg^{2+} ions*

Values were obtained from Figs. 3 and 4.

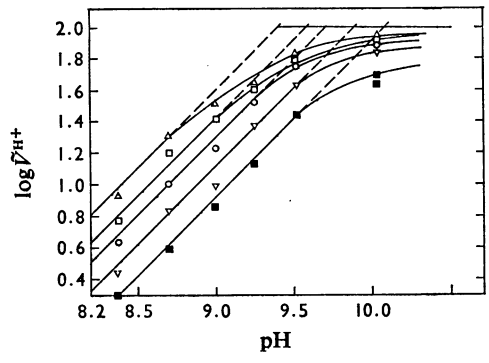


Fig. 8. *$\log\tilde{V}^{H+,Mg2+}$ versus pH plots for evaluation of the pK values of the ionizable groups of the enzyme–substrate–Mg^{2+} complex*

Values were obtained from Figs. 3 and 4. $[Mg^{2+}]$: ■, 0.4 μM; ▽, 1 μM; ○, 2.5 μM; □, 6.3 μM; △, 16 μM.

pH curve, which was obtained above, is only valid when $[Mg^{2+}]$ is much larger than K_{Mg}. Under these conditions, the functional group that binds Mg^{2+} is saturated. At lower Mg^{2+} concentration a shift of the $\log\tilde{V}^{H+}$ versus pH curve to higher pH values must occur, indicating competition between the H^+ and Mg^{2+} ions for the Mg^{2+}-binding functional group. This is as shown in Fig. 8, in which $\log\tilde{V}^{H+}$ versus pH is presented for Mg^{2+} concentrations of 0.4–16 μM. At 1 mM-Mg^{2+} we get the results shown in Fig. 1(*a*), which are identical with those obtained for $[Mg^{2+}] \to \infty$ obtained from intercepts of secondary plots of intercepts from, for example, Figs. 3 and 4 versus the reciprocal of the β-glycerophosphate concentration. In addition, Fig. 8 shows that at high pH values all $\tilde{V}$ values approximate to the same value independently of the Mg^{2+} concentration.

Evaluation of the pK value of the Mg^{2+}-binding functional group

From Fig. 7 the existence of a group dissociating at pH values above 10 and involved in the binding of Mg^{2+} is indicated. For the evaluation of this pK value, the following two competing equilibria have to be considered.

$$\frac{[H^+][EH_2]}{[EH_3]} = \tilde{K}_a''^{Mg2+}$$

(dissociation constant of enzyme–proton) (5)

$$\frac{[Mg^{2+}][EH_2]}{[H_2EMg]} = \tilde{K}^{H+}_{Mg}$$

(dissociation constant of enzyme–Mg^{2+}) (6)

Dividing eqn. (5) by eqn. (6) yields:

$$\frac{[H^+][H_2EMg]}{[Mg^{2+}][EH_3]} = \frac{\tilde{K}_a''^{Mg2+}}{\tilde{K}^{H+}_{Mg}} \quad (7)$$

A bend of the $\log\tilde{V}^{Mg2+}$ versus pH curve appears if the sum of the deprotonated forms of the enzyme $[H_2EMg]+[H_2E]$ is equal to the concentration of the protonated form $[EH_3]$. Since $[Mg^{2+}]$ is much higher than $[E]_t$ and the dissociation constant

$$K_{Mg} = \frac{[H_2E][Mg^{2+}]}{[H_2EMg]} \quad (8)$$

is very low (less than 1 μM), $[H_2E]$ is negligible compared with $[H_2EMg]$. Hence $[H_2EMg] = [EH_3]$. Under these conditions we find

$$\frac{[H^+]}{[Mg^{2+}]} = \frac{\tilde{K}_a''^{Mg2+}}{\tilde{K}^{H+}_{Mg}} \quad (9)$$

$[H^+]$ and $[Mg^{2+}]$ representing the points where bends occur, can be obtained from Fig. 8; $\tilde{K}^{H+}_{Mg}$ is obtained from Fig. 7. Hence $\tilde{K}_a''^{Mg2+}$ can be calculated from these data. The results are compiled in Table 2.

The following additional conditions are valid for the pK value of the Mg^{2+}-binding functional group:

$$[Mg^{2+}] = K_{Mg}$$

and

$$[H^+] = K_a'' \quad (10)$$

If we plot pMg and $p\tilde{K}^{H+}_{Mg}$ (from Table 2) versus pH (not shown), we find a common intercept with the co-ordinates pH = 10.15; pK_{Mg} = pMg = 6.35.

If $p\tilde{K}_a''^{Mg}$ and the pH at which the bend occurs are plotted as a function of pMg, a common intercept is obtained with the co-ordinates pMg = 6.35; pK_a'' = 10.15. Hence, the pK_a'' of the functional group is 10.15.

Reaction between enzyme, substrate, Mg^{2+} and H^+

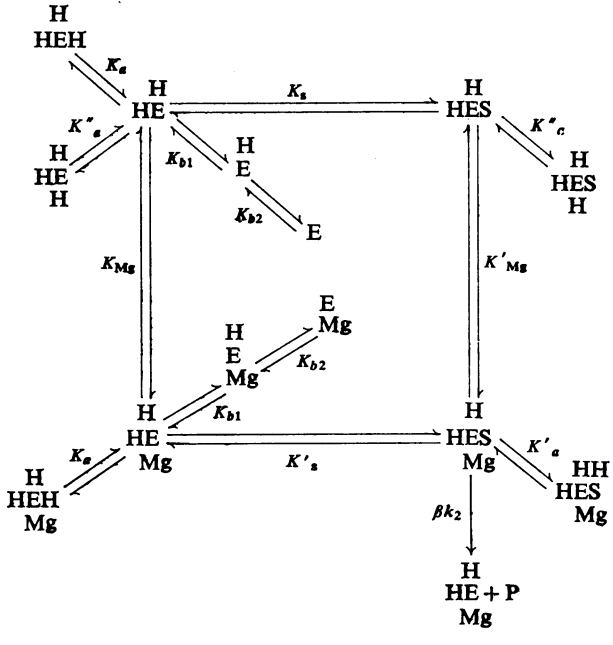
Figs. 3 and 4 show straight lines with a common intercept in the second quadrant near the abscissa. These and the other results indicate that the reported mechanism for the reaction of enzyme substrate and Mg^{2+} (Ahlers, 1974) is valid between pH8 and 10. This indicates that despite the fact that $\tilde{K}_s^{H+}$, $\tilde{K}_s'^{H+}$, $\tilde{K}_{Mg}^{H+}$ and $\tilde{K}_{Mg}'^{H+}$ change with the pH value, the following results are valid for the whole $[H^+]$ range. (1) There is no residual activity in the absence of Mg^{2+}, hence Mg^{2+} is an essential activator of the pig kidney alkaline phosphatase. (2) The binding of substrate occurs independently from the binding of activator; hence $\tilde{K}_{Mg}^{H+} = \tilde{K}_{Mg}'^{H+}$ and $\tilde{K}_s^{H+} = \tilde{K}_s'^{H+}$.

From Figs. 7 and 8 and from eqns. (9) and (10) the existence of a functional group, with a pK of 10.15, and involved in the process of Mg^{2+} binding, could be demonstrated. Since the dissociation constants of the groups responsible for substrate binding and conversion are known from the results given above, we can derive Scheme (3).

Table 2. *Dependence of the pH value of half-maximal velocity on the Mg^{2+} concentration and the corresponding values of $\tilde{K}_{Mg}^{H+}$ and $\tilde{K}_a''^{Mg2+}$*

The pH values and the Mg^{2+} concentrations were obtained from Fig. 8. The corresponding dissociation constants $\tilde{K}_{Mg}^{H+}$ can be calculated from Fig. 7. $\tilde{K}_a''^{Mg2+}$ was estimated by using eqn. 9.

$[Mg^{2+}]$ (μM)	pH	$[H^+]$ (pM)	$\tilde{K}_{Mg}^{H+}$ (μM)	$\tilde{K}_a''^{Mg2+}$ (pM)
16.0	9.40	400	1.2	30
6.3	9.58	260	0.87	36
2.5	9.70	210	0.72	57.5
1.0	9.885	130	0.56	73
0.4	10.15	71	0.435	77



Scheme 3. *Reaction between enzyme, substrate, Mg^{2+} and H^+ ions*

The dissociation equilibrium represented by the constant K_a' occurs for all enzyme species. Since it affects only the substrate conversion, it is only presented for EH_2MgS. The symbols $\overset{H}{HE}$ and $\overset{H}{HEH}$ indicate that the EH_3 species in one case is prevented from reacting with the activator $\left(\overset{H}{\underset{H}{HE}}\right)$ and in the other case from reacting with the substrate $\left(\overset{H}{HEH}\right)$. Hence in $\overset{H}{HES}$ the activator-binding site is occupied by a proton, and, in $\overset{HH}{HES}$ the substrate-converting residues are inactivated by protonation.

From Scheme (3) the following reaction rate equation can be derived for equilibrium conditions:

$$v = \frac{V[S]}{K'_m\left(1+\frac{[H^+]}{K_a}+\frac{K_b}{[H^+]}+\frac{K_b^2}{[H^+]^2}\right)\left[1+\frac{K_{Mg}(1+[H^+]/K''_a)}{[Mg^{2+}](1+[H^+]/K'_a)}\right]+[S]\left(1+\frac{[H^+]}{K'_a}\right)\left[1+\frac{K_{Mg}\cdot K'_m(1+[H^+]/K''_a)}{K_s\cdot[Mg^{2+}](1+[H^+]/K'_a)}\right]} \quad (11)$$

Eqn. (11) describes the dependence of the reaction rate v on the concentrations of substrate, activator and H^+. If $[Mg^{2+}]$ is much larger than K_{Mg} eqn. (11) simplifies to eqn. (1), whereas at constant $[H^+]$, eqn. (12) results, which has been given by Ahlers (1974):

$$v = \frac{V[S]}{K'_m\left(1+\frac{K_{Mg}}{[Mg^{2+}]}\right)+[S]\left(1+\frac{K'_m\cdot K_{Mg}}{K_s\cdot[Mg^{2+}]}\right)} \quad (12)$$

At $[S] \to \infty$ the reaction rate depends on the activator and H^+ concentration as follows:

$$v_{[S]\to\infty} = \frac{V}{\left(1+\frac{[H^+]}{K'_a}\right)\left[1+\frac{K_{Mg}\cdot K'_m(1+[H^+]/K''_a)}{K_s\cdot[Mg^{2+}](1+[H^+]/K'_a)}\right]} \quad (13)$$

This equation describes the results presented in Fig. 8. If $[S] \gg K_m$ and $[Mg^{2+}] \gg K_{Mg}$, we obtain

$$\tilde{V}^{H^+} = \frac{V}{1+[H^+]/K'_a} \quad (14)$$

which represents the pH-dependence of $\tilde{V}^{H^+}$. This equation describes the results presented in the upper curve of Fig. 8.

Functional groups in the active centre

The functional group with a pK of 7.0 could be an imidazole group of histidine or an α-amino group. In the case of the groups with p$K = 9.1$ and the group with p$K = 8.8$, one could be dealing with ε-amino groups of lysine and the co-ordinated water molecules on the Zn(II). As both pK values are very close together one cannot appoint them to one of the groups by pH measurements alone. As GSH is a non-competitive inhibitor and attacks the alkaline phosphatase at the Zn(II) site it is probable that the Zn(II)–water complex represents the group with the pK_a of 8.8 which catalyses the substrate splitting. The double-negatively charged substrate molecule could very easily be bound by two positively charged ε-amino groups of lysine which have a pK_b of 9.1. This agrees with Fishman & Ghosh (1967), who found a higher K_m value when the enzyme had reacted with amino group-specific reagents. This interpretation, however, is in contrast with that given by Fernley (1971), who discusses binding of the substrate at the Zn(II)–water complex. Appleton & Sarkar (1974) discussed a different mechanism for nucleophilic attack in connexion with carbonic anhydrase. They point out that the pK value for the loss of the last nitrogen-bound hydrogen of an imidazole residue co-ordinated to Zn(II) is depressed to a similar extent as the pH value of a water molecule co-ordinated to Zn(II). In the deprotonated form this imidazole residue can develop the nucleophile indirectly by accepting a water proton locally forming an OH^- ion. Such a mechanism might also be possible for alkaline phosphatase as an alternative to that discussed above.

The functional group that binds the Mg^{2+} ions has a pK value of 10.15. Hence this group could be a lysine, arginine or tyrosine residue. Since Mg^{2+} ions are bound very weakly by nitrogen atoms, but more strongly by oxygen atoms (Sigel & McCormick, 1970), the Mg^{2+}-binding residue may well be a phenolic hydroxyl group. This suggestion agrees well with inactivation studies of Hiwada & Wachsmuth (1974), who suggest that there is an essential tyrosine residue at the active centre of pig kidney alkaline phosphatase. In contrast with these results, Krishnaswamy & Kenkare (1970) could not detect dissociation of a phenolic hydroxyl group during their investigations of alkaline phosphatase of *E. coli*. However, these investigations were performed at pH values below 10. In addition, the *E. coli* enzyme is active in the absence of Mg^{2+} and hence this enzyme possibly lacks a Mg^{2+}-binding site in the active unit.

The role of Mg^{2+} in the process of substrate conversion is unclear. It is possible that Mg^{2+} ions react directly with an intermediate of the substrate; thus they might be designated as catalytic residues by the nomenclature of Koshland & Neet (1968). However, support for this suggestion is given neither in the literature nor by the results of this investigation. Hence it seems more likely that Mg^{2+} ions act as autosteric or regulatory effectors, as postulated by Brunel & Cathala (1973). This function would mean that the substrate could only be converted when Mg^{2+} had induced an active-site conformation in which the catalytic residues had obtained an optimum position relative to the substrate or its intermediates. Since pig kidney

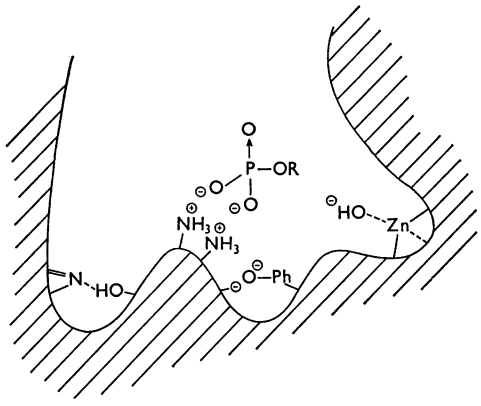


Fig. 9. *Model of the active centre of pig kidney alkaline phosphatase*

The five functional groups, derived from the results of the kinetic experiments, are shown in their active states. The non-protonated imidazole group stabilizes the substrate-binding centre by forming a hydrogen bond, e.g. with a hydroxyl group. The presence of an additional negative charge, involved in the binding of Mg^{2+}, is suggested.

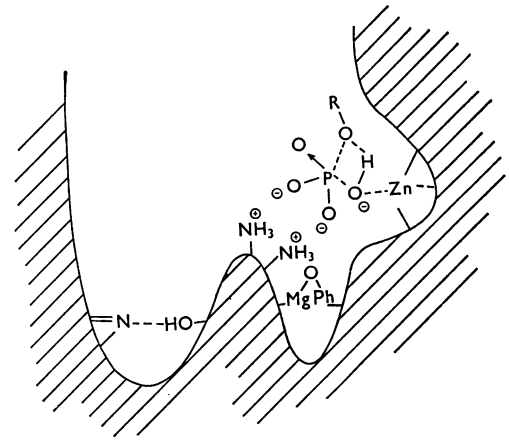


Fig. 10. *Substrate conversion at the active centre of pig kidney alkaline phosphatase*

Binding of Mg^{2+} to the 'autosteric site' leads to a conformational change of the active site, which causes a shift of the hydroxyl group, co-ordinated to the Zn(II), versus the phosphate monoester, which is bound to the ε-amino groups. The liberation of the alcohol residue occurs by nucleophilic attack by the hydroxyl group on the phosphorus atom.

alkaline phosphatase is inactive in the absence of Mg^{2+} ions, the influence of Mg^{2+} ions on the active site must be strong. This indicates that Mg^{2+} acts as an autosteric rather than a regulatory effector.

Active site of the pig kidney alkaline phosphatase

By using the results obtained by the above kinetic investigations, including varying the H^+, substrate and Mg^{2+} concentrations, a model for binding and converting β-glycerophosphate by the active site of pig kidney alkaline phosphatase is proposed (Figs. 9 and 10). In Fig. 9 the five functional groups derived are presented in their active form, as well as the binding of the substrate molecule. In addition, an unknown negative charge is inserted, which is involved in the binding process of Mg^{2+} ions. The imidazole group in the non-protonated state stabilizes the substrate-binding site by formation of a hydrogen bond.

The binding of Mg^{2+} leads (Fig. 10) to a conformational change, which causes the substrate to be shifted into the vicinity of the hydroxyl group, co-ordinated to the Zn(II). The hydroxyl groups liberates the alcoholic residue by nucleophilic attack on the phosphorus atom. The transfer of the phosphate group to a serine residue occurs coincidently; subsequently the enzyme is dephosphorylated. It should be mentioned that, by means of the kinetic methods applied here, differentiation between the phosphorylation and dephosphorylation steps is not possible. It is only possible to measure the rate-limiting step. Since there are hints that phosphorylation is the rate-limiting step at pH values above 7 (Trentham & Gutfreund, 1968; Fernley & Walker, 1969), we postulated that this step is governed by the functional groups of the active centre. The model proposed here could essentially also describe the dephosphorylation reaction. In this case, the phosphate group bound to the serine residue would be liberated after a conformational change induced by Mg^{2+} ions, caused by attachment of the hydroxyl group co-ordinated to Zn(II).

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